Virology 393 (2009) 228–237

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Interleukin-15 mediates potent antiviral responses via an interferon-dependent mechanism

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ARTICLE INFO

Article history:
Received 25 March 2009
Returned to author for revision 20 April 2009
Accepted 6 July 2009
Available online 3 September 2009

Keywords:
Interleukin-15
Interferons
NK cells
Recombinant vaccinia virus

ABSTRACT

Interleukin-15 (IL-15) is a potent growth factor for activated T and natural killer (NK) cells, stimulator of memory T cells and plays an important role in viral immunity. To investigate mechanisms underlying the antiviral activity of IL-15, a recombinant vaccinia virus (rVV) encoding murine IL-15 (VV-IL-15) was constructed. Following infection of mice with VV-IL-15, virus titres in the ovaries were significantly reduced compared to mice infected with control VV. Growth of VV-IL-15 was also reduced in nude athymic mice, indicating the antiviral activity of IL-15 does not require T cells. Additionally, VV-IL-15 augmented the cytolytic activity of natural NK cells in the spleen and enhanced interferon (IFN) mRNA expression and transcription factors associated with IFN induction. Using knockout mice and antibody depletion studies, we showed for the first time that the control of VV-IL-15 replication in mice is dependent on NK cells and IFNs and, in their absence, the protective role of IL-15 is abolished.

Introduction

The early stages of viral infection are controlled by the induction of non-specific immune cells including natural killer (NK) cells, dendritic cells and macrophages, and by the production of cytokines including interferons (IFNs). Cytokines and chemokines are largely produced by T cells and macrophages and they control multiple arms of the antiviral immune response (Ramshaw et al., 1997; Mahalingam et al., 2001c).

Interleukin-15 (IL-15) is a cytokine with antiviral activity, sharing a number of key functions with IL-2 (Grabstein et al., 1994). Both IL-2 and IL-15 have a similar tertiary structure and belong to the four alpha-helix bundle family of cytokines. Although IL-15 shares common receptor subunits with IL-2 for signal transduction (the IL-2Rα and IL-2R subunits), each cytokine utilises a different receptor subunit for high-affinity binding (Tagaya et al., 1996). The common biological functions of IL-2 and IL-15 include stimulation of NK cells, induction of T and B cell proliferation, and the stimulation of cytokine secretion (Armitage and Alderson, 1995; Carson et al., 1994; Waldmann and Tagaya, 1999). Although IL-15 shares many of the biological effects of IL-2, significant differences exist at the molecular and cellular level between these two cytokines (Fehniger and Caligiuri, 2001; Waldmann et al., 2001). Exogenous administration of IL-15 has been reported to regulate NK cell survival and stimulate IFN production by NK cells (Carson et al., 1994; Puzanov et al., 1996). Studies in knockout mice have demonstrated that IL-15 is required for the development and survival of NK cells and both memory and activated $CD8^+$ T cells (Kennedy et al., 2000; Lodolce et al., 1998).

The presence of a virus-inducible region in the IL-15 promoter suggests IL-15 is a component of host antiviral defense mechanisms (Azimi et al., 2000). This has been confirmed for infection with a number of viruses including herpes simplex virus-1 (HSV-1), HSV-2 and vaccinia virus (VV) (Ahmad et al., 2000; Perera et al., 2001; Tsunobuchi et al., 2000). IL-15 deficient mice are unable to clear VV infection (Kennedy et al., 2000), although these mice lack NK cells, making interpretation of results difficult. Landmark studies by Perera et al. (2001) using a rVV encoding human IL-15 showed a direct antiviral effect of IL-15. The rVV encoding IL-15 was cleared more rapidly from mice than the corresponding control virus (Perera et al., 2001). The mechanisms of protection in mice infected with rVV expressing IL-15 were not established, although protection was associated with increased NK cell numbers and IFN-γ production.

In this study, we sought to identify specific mechanisms of IL-15-driven antiviral activity by investigating mechanisms of clearance of rVV encoding IL-15 in mice. For these studies we developed rVV encoding murine IL-15 (mIL-15). This differs from previous studies which used human IL-15, and may be an important enhancement since there is only 73% and 54% amino acid homology between the human and mouse IL-15 gene (Anderson et al., 1995a) and receptor

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doi:10.1016/j.virol.2009.07.030

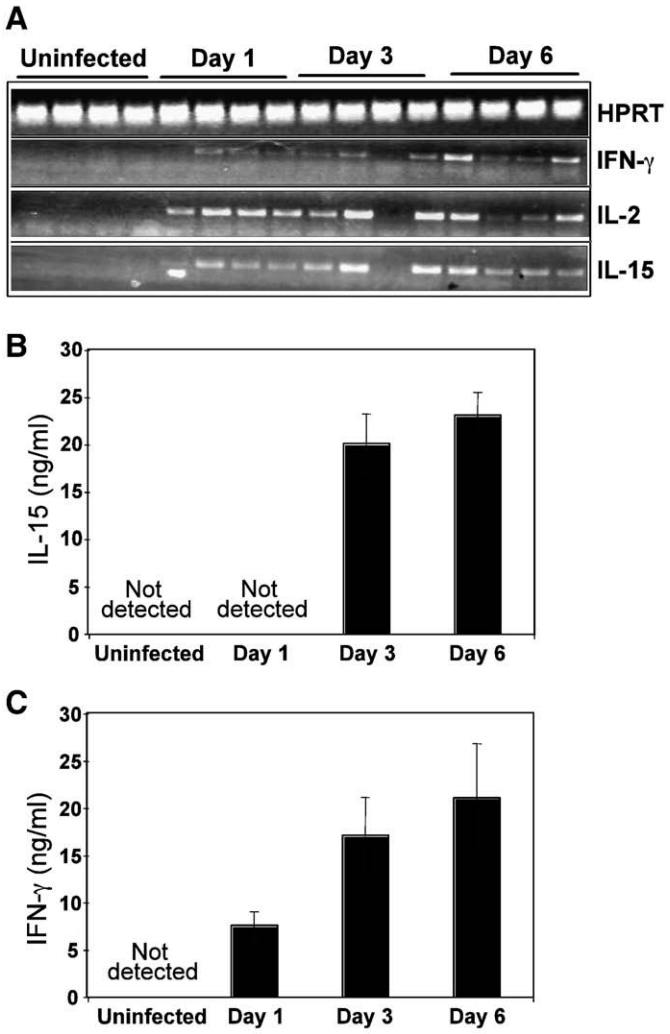

Fig. 1. IL-15 mRNA and protein expression *in vivo*. (A) Time course of expression of IL-15, IL-2, IFN-γ and the housekeeping gene HPRT mRNA in C57BL/6 mice following infection with VV-WR. Total RNA was prepared from the spleens of groups of four uninfected mice and infected mice at days 1, 3 and 6 post infection (p.i). (B and C) Time course of IL-15 and IFN-γ protein production in C57BL/6 mice following infection with VV-WR. Spleens from groups of three uninfected mice and infected mice at days 1, 3 and 6 p.i. and were homogenized in PBS. Cytokine concentrations in the supernatants were determined by ELISA.

(Anderson et al., 1995b), respectively. Furthermore, differences in signaling potency between human and mouse IL-15 through the IL-15 receptor have been observed (Eisenman et al., 2002).

Virally encoded IL-15 enhanced viral clearance and prevented mortality in athymic nude mice. The clearance of VV-IL-15 was found to be mediated by increased levels of NK cell cytolytic activity and by the induction of IFNs and associated transcription factors. We show, for the first time, that the protective role of IL-15 against VV infection is dependent on the presence of IFNs.

Results

IL-15 is expressed early following VV infection and parallels IL-2 and IFN-γ expression

IL-15 mRNA expression was measured during VV infection to gain insight into potential roles for IL-15 in antiviral immunity. Spleens were removed from VV-infected C57BL/6 mice at days 1, 3, 6 10, 12 and 14 post infection (p.i). IL-15 mRNA was absent in uninfected mice, but rapidly upregulated following infection, with expression being detectable by day 1, and peaking by day 3 (Fig. 1A). The expression of IL-15 mRNA decreased rapidly after day 6, a timepoint that correlates with virus clearance. IL-15 mRNA was not detected at days 10, 12 and 14 (data not shown). The kinetics of IL-15 mRNA expression was similar to that observed for two known antiviral cytokines IL-2 and IFN-γ (Fig. 1A).

In order to determine that the mRNA profile reflected protein expression, ELISAs for IL-15 and IFN-γ were performed on supernatants of homogenized spleen tissues. Consistent with the mRNA data, at various time points p.i., IL-15 protein correlated with IFN-γ protein and peaked at day 6 (Figs. 1B, C). Since the kinetics of IL-15, IFN-γ and IL-2 were similar, and both IFN-γ and IL-2 have previously been shown to play important roles in the clearance of poxviruses, the clear implication was that murine IL-15 may mediate similar functions.

VV-IL-15 replication is attenuated in vivo

The role of IL-15 in mediating viral clearance *in vivo* was investigated by constructing a rVV encoding mouse IL-15 (VV-IL-15). Following infection of 143B cells with VV-IL-15, we were able to confirm that the infected cells produced IL-15 (Fig. 2A) and that this IL-15 was fully bioactive (Fig. 2B). IL-15 could not be detected in cultures infected with VV-X.

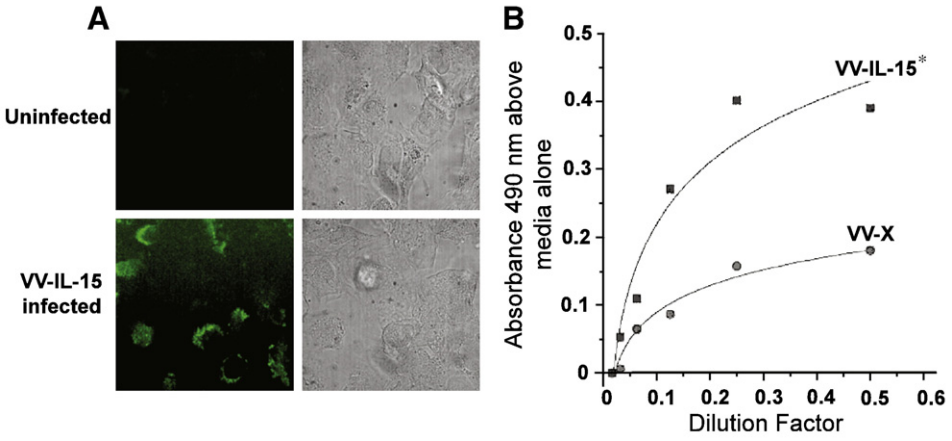

Fig. 2. Protein expression by VV-IL-15. (A) Confocal images of fixed, permeabilised cells infected with VV-IL-15 and stained with murine IL-15 antibody and FITC-conjugated secondary antibody. Uninfected cells stained with both antibodies were used as a control. Transmission images of the same fields are shown on the right. (B) CTLL-2 cell proliferation in response to two-fold dilutions of concentrated protein harvested from VV-infected cells. Cell proliferation is proportional to the absorbance at 490 nm. * indicates VV-IL-15 is significantly higher than VV-X ($p<0.0001$).

Table 1
Mortality of rVV-infected Swiss, nude mice.

Virus	10^4 pfu	10^5 pfu	10^6 pfu	10^7 pfu
VV-X	12.5%	25%	100% (10.8 ± 0.2[a])	100% (6.5 ± 0.2[a])
VV-IL-15	NM	NM	NM	NM
VV-IL-2	NM	NM	NM	NM

Groups of 7 mice were observed for 45 days.
NM: no mortality observed.
[a] The mean time to death (days) ± SEM is given for groups with 100% mortality.

A single-step growth experiment was performed in CV-1 cells to determine the *in vitro* replication of the IL-15 encoding virus compared to the control virus. The pattern of viral infectivity was similar to the control virus over a period of 48 h before cell lysis occurred (data not shown). This indicated that insertion and over-expression of IL-15 by rVV neither affected the replication of the vaccinia virus nor attenuated the virus *in vitro*.

Athymic Swiss outbred nude mice, which lack mature T cells, were infected with rVV to investigate the effects of virus-expressed IL-15 *in vivo*. Nude mice were highly susceptible to infection with VV-X, showing 100% mortality at the higher doses (10^6–10^7 pfu), and 12.5% mortality at 10^4 pfu, the lowest dose tested. Mice infected with the higher doses of VV-X developed severe pox lesions during the first week of infection and demonstrated severe weight loss as well as morbidity prior to death. In contrast, nude mice survived infection with VV-IL-15 at all doses tested, with minimal weight loss and morbidity (Table 1). For comparison, nude mice were infected with VV-IL-2, a previously described rVV known to be highly attenuated through its ability to stimulate strong NK- and T cell responses. Similar to VV-IL-15, all mice infected with VV-IL-2 successfully cleared VV infection. These results highlight the potent antiviral function of IL-15, even in the absence of T cells.

To further assess the antiviral effects of IL-15, we investigated the growth of rVV in ovaries of nude and wild-type mice. The ovary was chosen for these studies since VV replicates to high titres in this organ. Nude mice infected with VV-IL-15 exhibited markedly lower viral titers compared to mice infected with VV-X at all time points tested ($p<0.01$) (Fig. 3A). By day 1 post infection, there was already a 100-fold reduction in virus titres in mice infected with VV-IL-15, indicating that the antiviral activity of IL-15 is relatively rapid. The degree of attenuation of VV-IL-15 in immunocompetent mice was similar to that observed in nude mice (Fig. 3B). Although it is difficult to make direct comparisons between outbred nude mice and C56BL/6 mice, the results indicate that a substantial component, if not all, of the antiviral activity of the virus-encoded IL-15 is T cell independent.

In these experiments, additional groups of mice were infected with VV-IL-2, a virus that is highly attenuated due to heightened activity of both NK cells and T cells. Using this comparison, we conclude that IL-15 and IL-2 have comparable antiviral activity, although IL-15 may be slightly less potent (Figs. 3A, B).

Histological analysis of stained ovarian sections revealed extensive damage to the stromal tissue and follicles of ovaries in C57BL/6 mice infected with VV-X (Fig. 4B). In mice infected with VV-IL-15 minimal damage to the ovaries was observed (Fig. 4D), consistent with reduced levels of virus replication in the ovary. Ovarian sections from VV-IL-2-infected mice (Fig. 4C) displayed minimal histopathological changes and were similar to ovarian sections from uninfected control mice (Fig. 4A).

Splenic NK cytolytic activity is enhanced in VV-IL-15-infected mice

It is clear that early immune mechanisms play a role in the clearance of VV-IL-15, since virus load was dramatically reduced as early as day one in mice infected with VV-IL-15 compared to VV-X. Several studies have implicated IL-15 in NK cell activation (Biron and Brossay, 2001), and we hypothesized that increased NK cell cytolytic activity contributed to the enhanced clearance of VV-IL-15. Splenic NK cytolytic activity was measured in groups of uninfected mice and mice infected with VV-X and VV-IL-15. In some experiments VV-IL-2 was included as an additional control based on the ability of this virus to stimulate high levels of NK activity (Karupiah et al., 1990). In both nude and wild-type mice, NK cell cytolytic activity at day 1 p.i. was approximately 3-fold higher in mice infected with VV-IL-15 than with VV-X (Figs. 5A, C; $p<0.05$). The high NK cytolytic activity was not due to IL-15-induced proliferation of NK cells as there was no increase in NK cell numbers in mice infected with VV-IL-15 (data not shown). By day 3 there was little or no difference in NK cell activity between mice infected with VV-X or VV-IL-15 (Figs. 5B, D). The heightened NK activity at day 1 following infection with VV-IL-15 correlates well with the rapid clearance of this virus (Fig. 3A), suggesting that enhanced NK cell activity may underlie the antiviral activity of virus-encoded IL-15. The NK cell activity in mice infected with VV-IL-15 was generally less than that in mice infected with VV-IL-2 (Figs. 5B, C, D), suggesting that the ability of IL-15 to promote NK cell cytotoxicity may be less than that of IL-2.

IFNs, IRF-1 and NF-κb are upregulated in VV-IL-15-infected mice

IL-15 can induce the production of antiviral type I and type II IFNs in NK cells, monocytes, macrophages and dendritic cells *in vitro* (Carson et al., 1999; Jinushi et al., 2003; Liu et al., 2004). Therefore, the role of IFNs in VV-IL-15-mediated protective responses was

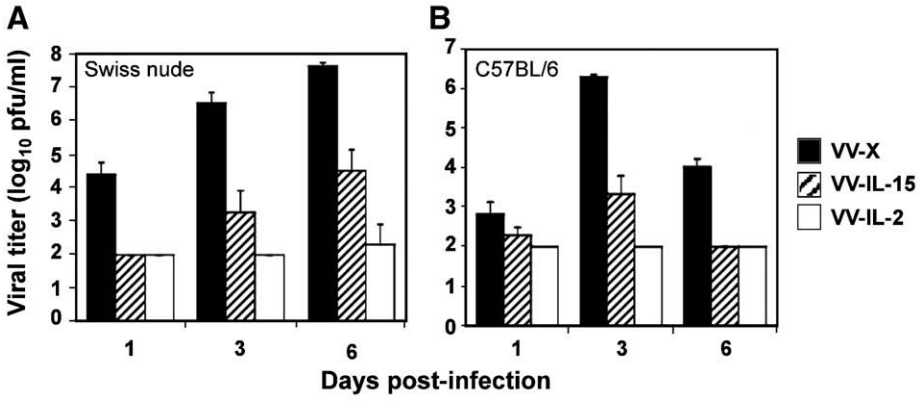

Fig. 3. Growth kinetics of rVV replication in ovaries of VV-infected Swiss nude mice (A) and C57BL/6 mice (B). Groups of 4 female mice were infected intravenously with 10^6 pfu (Swiss nude) or 10^7 pfu (C57BL/6) rVV. Ovaries were collected from the mice on days 1, 3 and 6 and the viral titers determined. Shown is the mean plus standard error of the mean. The limit of detection of the assay is 2 $\log_{10}$ pfu/ml. At all timepoints the mean viral titers of the Swiss nude and C57BL/6 mice infected with VV-X were significantly higher than mice infected with VV-IL-15 and VV-IL-2 (one-way ANOVA, $p<0.01$).

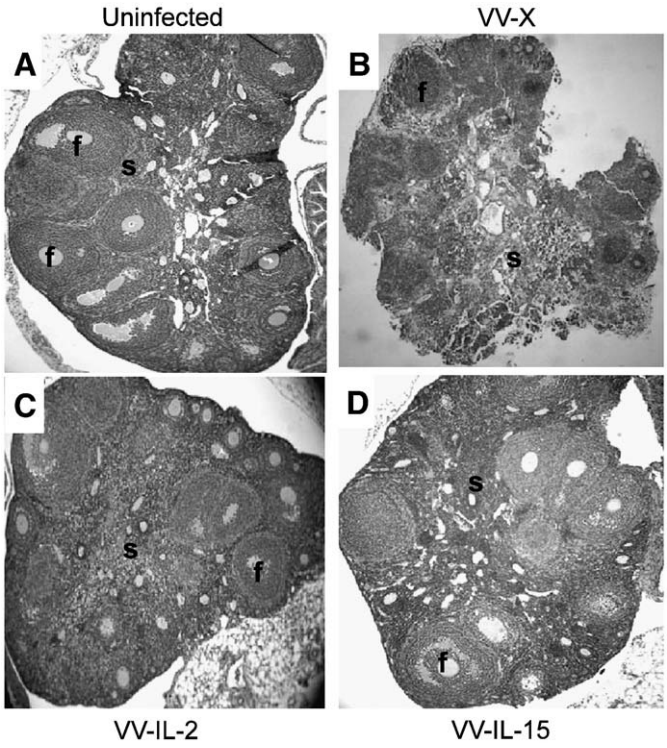

Fig. 4. Histological examination of ovaries from C57BL/6 mice infected with rVV. Ovarian sections were harvested 6 days after mice were infected with 10^7 pfu of rVV and stained with haematoxylin and eosin. Extensive damage to the stromal tissue (S) and follicles (F) was evident in VV-X (B) infected ovaries. However, minimal histopathological changes were observed in ovarian sections of VV-IL-2 (C) and VV-IL-15 (D) infected mice, compared to an uninfected control (A). Images are at 100× magnification.

investigated. Groups of C57BL/6 mice were infected with rVV, sacrificed on day 3 and the spleens collected for analysis of IFN mRNA expression and protein. Virus-expressed IL-15 induced considerably higher levels of IFN-β, IL-15, IFN-γ and IRF-1 mRNA than in mice infected with VV-X (Fig. 6A). In addition, IL-15 and IFN-γ protein levels were significantly higher in VV-IL-15-infected mice compared to control virus-infected animals (Figs. 6B, C; $p<0.01$). A similar trend was seen in the ovaries with higher levels of IFN-γ protein detected in VV-IL-5 infected mice compared to VV-X infected mice at 3 days after infection (data not shown). In a separate experiment, the transcription factors IRF-1 and NF-κB were examined. These transcription factors bind to a common motif in the promoter region of the genes for IFNs and several IFN-inducible genes. IRF-1 and NF-κb levels were higher in the spleens of VV-IL-15 infected mice than in the spleens of mice infected with the control virus, VV-X (Figs. 6D, E). Taken together, these results provide clear evidence of heightened IFN activity in response to virus-encoded IL-15, and suggest a further mechanism of IL-15-mediated antiviral activity.

In vivo NK cell depletion partially reverses the antiviral activity of virus-encoded IL-15

In order to establish whether NK cells contributed to the control of VV-IL-15 replication, mice were depleted of NK cells using a monoclonal antibody (PK136) to $NK1.1^+$ cells (Koo et al., 1986). Administration of PK136 to mice infected with VV-X resulted in a severe reduction in NK cytolytic activity (data not shown), thus confirming treatment efficacy. In mice infected with VV-X, depletion of NK cells had no effect on virus load in the ovaries (Fig. 7A), and a moderate increase in the spleen (Fig. 7B). In contrast, virus load in the ovary and spleen of VV-IL-15 infected mice was significantly higher following NK cell depletion (Fig. 7B). However, NK cell depletion in VV-IL-15 infected mice did not fully abrogate the attenuation of VV-IL-15, since the titres in VV-IL-15 infected mice did not return fully to the levels in VV-X-infected mice. These results indicate that NK cells are major contributors to the antiviral activity of virus-encoded IL-15, but that additional factors may also participate.

We speculated that NK cells were also responsible for the enhanced expression of IFNs in mice infected with VV-IL-15. In the spleens of mice infected with VV-X or VV-IL-15, depletion of NK cells markedly reduced expression of both IFN-γ and IFN-β (Figs. 7C, D). This result suggested a potential mechanism for the antiviral activity of NK cells in VV-IL-15 infected mice through the production of IFNs. However, NK cell depletion did not fully block IFN mRNA expression, especially for IFN-β, suggesting additional sources of IFN production.

The absence of IFNs abolish the protective role of IL-15

To test the role of IFNs in the antiviral activity of virus-encoded IL-15, we employed a range of IFN- and IFN receptor knockout mice. Following infection with VV-X, mice deficient in IFN-γ or the IFN-γ receptor showed 100% mortality. Similarly, mice deficient in the IFN-

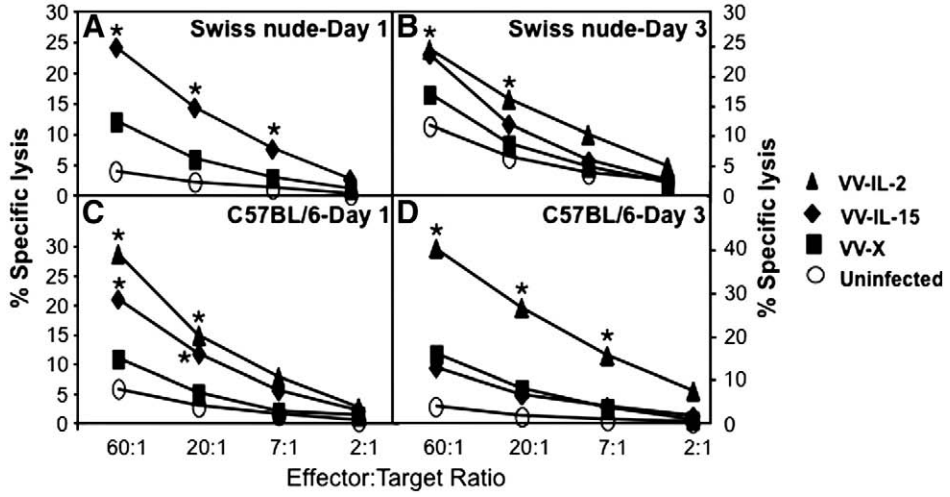

Fig. 5. Lysis of YAC-1 target cells by splenocytes from Swiss nude mice (A and B) and C57BL/6 mice (C and D). Natural killer cell cytolytic activity was measured on days 1 and 3 post infection using spleen cells from VV-infected mice. Uninfected mice were used as controls. Data shown are the mean lysis values from 4 mice in each group. SEM values were less than 5% and have been omitted for clarity. Asterisks indicate that NK cytolytic activity was significantly higher in VV-IL-15- or VV-IL-2-infected mice compared to mice infected with VV-X (one-way ANOVA, $p<0.01$).

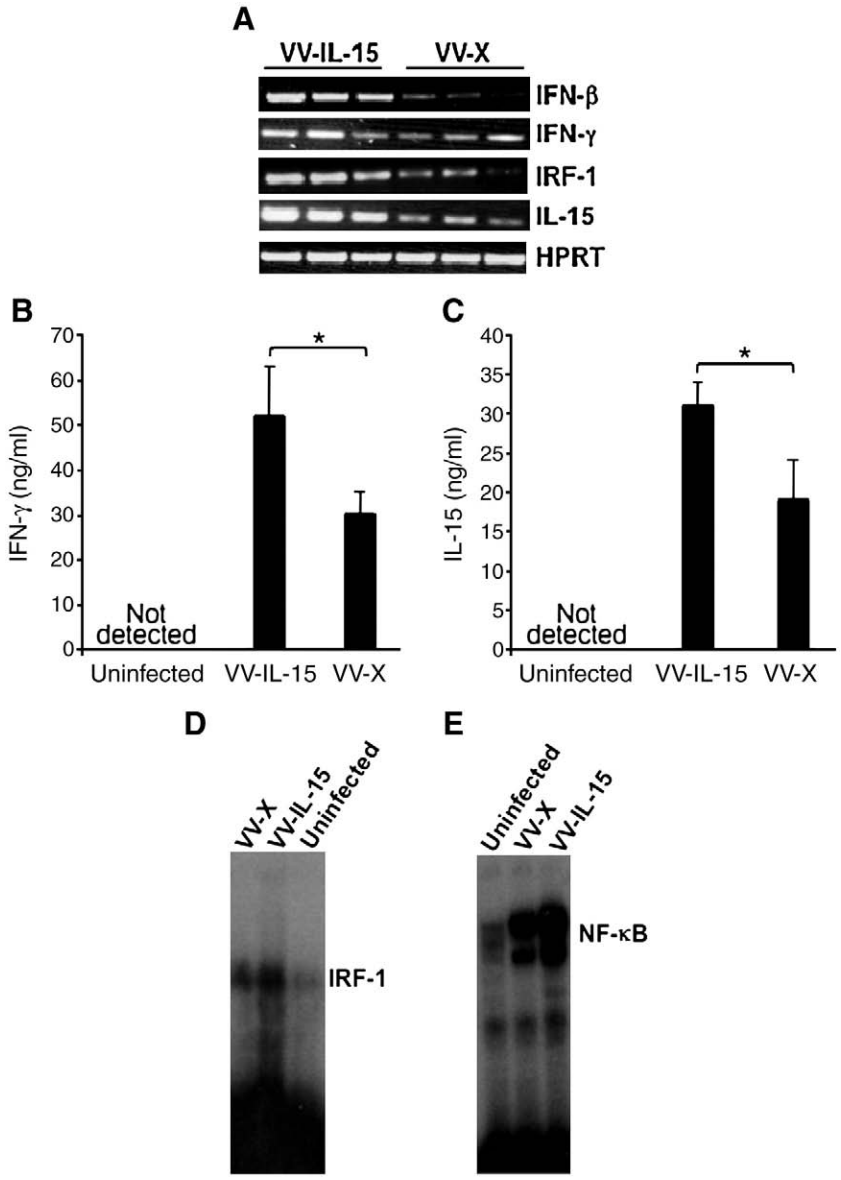

Fig. 6. Analyses of cytokines and transcription factors *in vivo*. (A) Expression of IFN-γ, IFN-β, IRF-1, IFN-15 and the housekeeping gene HPRT mRNA transcripts in the spleens of groups of 3 C57BL/6 mice three days post infection with VV-IL-15 and VV-X. (B and C) IL-15 and IFN-γ protein levels in the spleens of groups of 3 C57BL/6 uninfected mice and mice infected with VV-IL-15 or VV-X (three days post infection). Cytokine concentrations in the supernatants were determined by ELISA. Electrophoretic mobility shift assay showing IRF-1 and NF-κB activity in nuclear extracts prepared from the spleens of uninfected mice and mice infected (three days post infection) with recombinant vaccinia viruses.

α/β receptor, and mice deficient in both the IFN-α/β and IFN-γ receptor, were fully susceptible to VV-X infection (Table 2). These results confirm the importance of both type I and type II IFN in control of VV infection. In contrast, IFN-$\gamma^{-/-}$, IFN-$\gamma^{-/-}$ R, and IFN-α/β $R^{-/-}$ mice were fully resistant to infection with VV-IL-15. Only mice lacking both IFN-γ receptor and IFN-α/β receptor were susceptible to VV-IL-15 infection, showing 100% mortality (Table 2).

Virus load in the spleen and ovaries of IFN-deficient mice 3 days following rVV infection was also measured. VV-IL-15 grew to significantly higher titres in IFN-$\gamma^{-/-}$ and IFN-$\alpha/\beta^{-/-}$ R mice than in wild-type mice, although in neither deficient mouse strain did the virus load approach that of mice infected with VV-X (Table 3). In IFN-α/β, γ $R^{-/-}$ mice the virus load in the ovaries and spleen was higher than in mice deficient in either IFN-γ or IFN-α/β signaling, and was comparable to the virus load found in mice infected with VV-X (Table 3). These results were confirmed using neutralizing antibodies to IFN-γ and IFN-α/β. Neutralization of either IFN resulted in enhanced virus load in mice infected with VV-IL-15 (Table 4). The virus load in VV-IL-15-infected mice treated with neutralizing antibodies to both IFN-γ and IFN-α/β was higher still, and similar to that found in mice infected with VV-X. Taken together, the results suggest that both IFN-γ and IFN-α/β contribute to the enhanced clearance of VV-IL-15, and that in combination the IFNs largely account for the antiviral activity of virus-encoded IL-15.

Discussion

IL-15 is involved in protection against several viral infections, including human herpes viruses, HIV and influenza virus (Ahmad et al., 2000; Chehimi et al., 1997; Fawaz et al., 1999). Most of these studies were performed *in vitro* using human peripheral blood mononuclear cells (PBMCs) infected with the relevant virus. A few studies have shown that IL-15 has antiviral activity *in vivo* (Ashkar and Rosenthal, 2003; Perera et al., 2001; Tsunobuchi et al., 2000). The ability of IL-15 to promote NK cell activation is a major factor in the antiviral activity of this cytokine (Fawaz et al., 1999). However, IL-15-

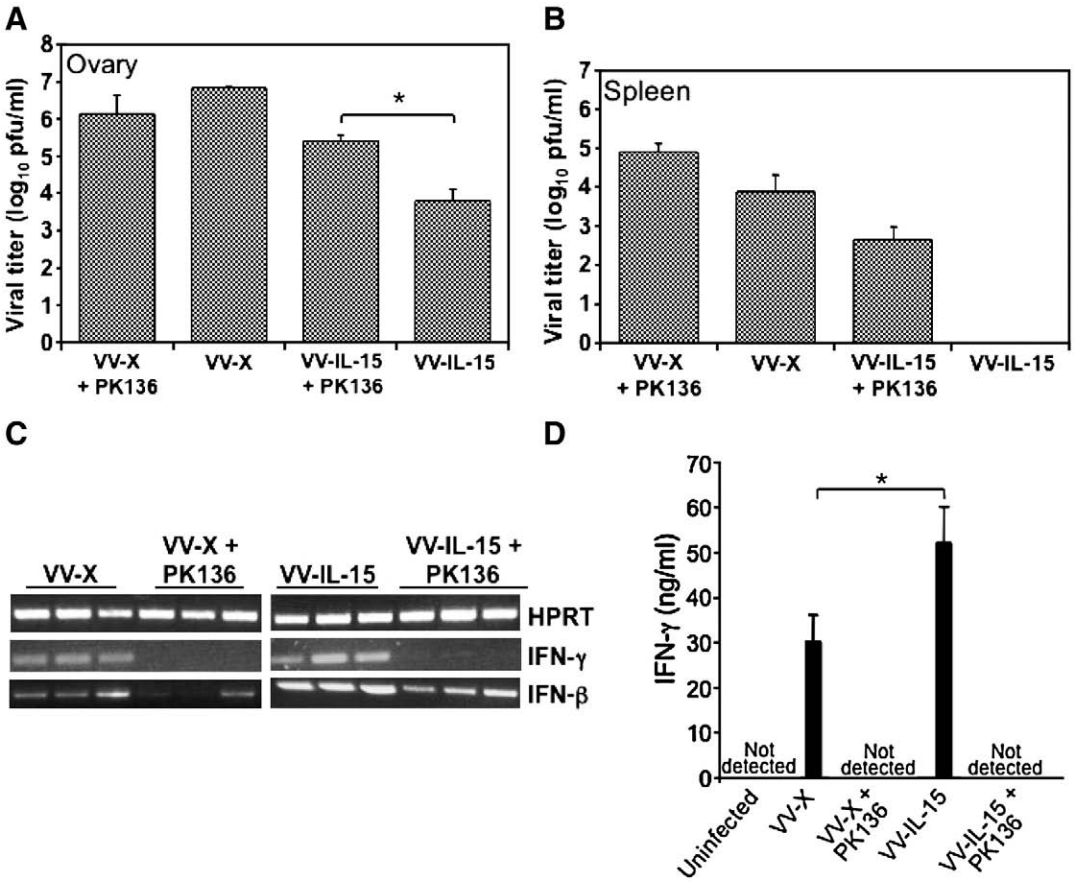

Fig. 7. Effects of natural killer (NK) cell depletion on viral titers and IFN levels. Groups of 4 female C57BL/6 mice were depleted of NK cells by intraperitoneal administration of the PK136 monoclonal antibody on days −1 and 1 and infected on day 0 with 10^7 pfu VV-X or VV-IL-15. Ovaries (A) and spleens (B) were harvested on day 3 and the viral titers determined. The standard error of the mean for each group is represented as error bars. * indicates a significant difference between the groups. (C) Expression of IFN-γ, IFN-β and the housekeeping gene HPRT in the spleens of groups of three NK depleted and non-depleted mice three days post infection with rVV. (D) IFN-γ protein levels in the spleens of groups of 3 C57BL/6 uninfected mice, NK depleted and non-depleted mice three days post infection with rVV. Cytokine concentrations in the supernatants were determined by ELISA (*$p<0.05$).

mediated antiviral activity independent of NK cells has also been reported, indicating that IL-15 has additional antiviral functions (Gill et al., 2005). Clearly the mechanisms for IL-15-mediated antiviral effects are not fully understood and warrant further investigation. An early landmark study used rVV expressing human IL-15 to demonstrate potent antiviral function for IL-15 *in vivo* (Perera et al., 2001). Here we have extended these findings, first by identifying key cells and cytokines involved in the antiviral activity of IL-15, and second, by using an rVV expressing mouse (rather than human) IL-15 to more accurately model the function of this cytokine *in vivo*. In particular, we demonstrate that the antiviral effect of IL-15 during a generalized vaccinia virus infection *in vivo* is dependent on the presence of IFNs. To the best of our knowledge this is the first study to demonstrate a requirement for IFNs in IL-15-mediated antiviral activity *in vivo*.

Table 2
Mortality of knockout and wild-type mice following infection with VV-IL-15 or VV-X.

Mouse strains	VV-IL-15	VV-X
	% mortality (MTD)*	% mortality (MTD)*
WT (129/SvEv)	NM	62.5%
IFN-α,β,γ $R^{-/-}$ (129/SvEv)	100% (4.8 ± 0.4*)	100% (3.9 ± 0.3*)
IFN-α,β $R^{-/-}$ (129/SvEv)	NM	100% (13.8 ± 0.9*)
IFN-γ $R^{-/-}$ (129/SvEv)	NM	100% (25 ± 1.3*)
WT (C57BL/6)	NM	50%
IFN-γ (C57BL/6)	NM	100% (26.6 ± 1.1*)

Groups of 8 mice were infected intravenously with 10^7 pfu of VV and observed for 50 days.
NM: no mortality observed.
*The mean time to death (days) ± SEM is given for groups with 100% mortality.

Similar to the study by Perera et al. (2001), we found that the antiviral activity of IL-15 was expressed rapidly following infection of mice with VV-IL-15. Taken together with the very early expression of IL-15 during VV infection (Fig. 1), these data indicate that IL-15 could play an important role in the early immune response to virus infection. This observation is consistent with other studies demonstrating the early activity of IL-15 in resolving infections. For example, infecting mice with intracellular bacteria such as Mycobacteria (Doherty et al., 1996), Listeria (Hirose et al., 1998) and HSV-2 (Gill

Table 3
Replication of rVV in organs of IFN knockout mice.

Virus and mouse strains	Spleens (Log_{10} virus titers ± SEM)	Ovaries (Log_{10} virus titers ± SEM)
VV-X		
WT (129/SvEv)	3.0 ± 0.1	6.6 ± 0.2
IFN-γ $R^{-/-}$ (129/SvEv)	4.5 ± 0.13	6.3 ± 0.1
IFN-α,β $R^{-/-}$ (129/SvEv)	4.3 ± 0.5	7.1 ± 0.2
IFN-α,β,γ $R^{-/-}$ (129/SvEv)	4.8 ± 0.4	7.7 ± 0.1
VV-IL-15		
WT (129/SvEv)	No virus detected	4.8 ± 0.11
IFN-γ $R^{-/-}$ (129/SvEv)	3.1 ± 0.06[a]	6.7 ± 0.08
IFN-α,β $R^{-/-}$ (129/SvEv)	3.5 ± 0.2[a]	6.5 ± 0.1
IFN-α,β,γ $R^{-/-}$ (129/SvEv)	4.7 ± 0.15[b]	7.0 ± 0.11

Groups of 4 mice were infected intravenously with 10^7 pfu of VV and spleens and ovaries were collected 3 days post infection to determine viral titers. The limit of detection of the assay is 2 log_{10} pfu/ml.
[a] $p<0.05$, compared to IFN-γ $R^{-/-}$ or IFN-α/β $R^{-/-}$ mice infected with VV-X.
[b] $p<0.05$, compared to IFN-γ $R^{-/-}$ or IFN-α/β $R^{-/-}$ mice infected with VV-IL-15.

Table 4
Effect of treatment with antibodies to IFN-γ or IFN-α/β or both on rVV replication in C57BL/6 mice.

Virus and treatment	Spleens (Log_{10} virus titers ± SEM)	Ovaries (Log10 virus titers ± SEM)
VV-X		
+ GL113	2.7 ± 0.2	5.9 ± 0.4
+ anti-IFN-γ	3.9 ± 0.3	6.1 ± 0.2
+ anti-IFN-α/β	3.6 ± 0.4	6.5 ± 0.3
+ anti-IFN-γ + anti-IFN-α/β	4.3 ± 0.4	7.2 ± 0.4
VV-IL-15		
+ GL113	No virus detected	4.2 ± 0.1
+ anti-IFN-γ	2.8 ± 0.1[a]	5.8 ± 0.5
+ anti-IFN-α/β	2.9 ± 0.3[a]	6.0 ± 0.4
+ anti-IFN-γ + anti-IFN-α/β	3.9 ± 0.5[b]	6.9 ± 0.2

Groups of 3 C57BL/6 mice were treated with antibodies to IFN-γ, IFN-α/β, IFN-γ + IFN-α/β or the irrelevant control GL113 and infected intravenously with 10^7 pfu virus. Mice were killed 3 days post infection and virus titers in the spleen and ovaries determined.
[a] $p<0.05$, compared to VV-X infected mice depleted of either IFN-γ or IFN-α/β.
[b] $p<0.05$, compared to VV-IL-15 infected mice depleted of either IFN-γ or IFN-α/β.

et al., 2005; Tsunobuchi et al., 2000) induced the early production of IL-15 that was necessary for protection against these pathogens.

NK cells are key contributors to the early antiviral response through their cytolytic activity and production of soluble mediators (Biron and Brossay, 2001). Previous studies have shown a strong association between IL-15 and NK cell activity (Carson et al., 1994; Perera et al., 2001; Puzanov et al., 1996), confirmed by our findings of enhanced NK activity in mice infected with VV-IL-15. The increased NK activity represents a major antiviral mechanism for IL-15, since depletion of NK cells markedly reversed the attenuation of VV-IL-15. However, VV-IL-15 titers in NK depleted mice were still lower than in mice infected with VV-X, especially in the spleen, suggesting that additional early immune components may contribute to the antiviral effects of IL-15.

IL-15 can stimulate production of IFNs from a range of immune effector cells (Carson et al., 1999; Jinushi et al., 2003; Liu et al., 2004), and we hypothesized that IFNs could mediate the antiviral activity of IL-15. Infection with VV-IL-15 resulted in elevated expression of IFNs and the IFN-related transcription factors NF-κB and IRF-1, and represents a highly plausible mechanism for the antiviral activity of rVV encoded IL-15. The majority of IL-15-mediated IFN production could be attributed to NK cells, since depletion of NK cells resulted in complete reduction in IFN-γ mRNA expression, and substantial reduction in IFN-β mRNA expression. Based on these results we conclude that non-NK cells also contribute to the elevated IFN production following infection with VV-IL-15. IL-15-mediated production of type I IFNs may represent a key step in the rapid amplification of NK cell function since type I IFNs can themselves enhance NK cell activation during VV infection (Martinez et al., 2008).

Using IFN-deficient mice, and neutralizing antibodies, we demonstrated that the antiviral activity of virus-encoded IL-15 required the activity of both IFN-α/β and IFN-γ. Mice lacking receptors for IFN-α/β or IFN-γ were highly susceptible to infection with VV-X, but were partially resistant to infection with VV-IL-15. In contrast, mice lacking receptors for both IFN-α/β and IFN-γ were highly, and equally, susceptible to VV-X and VV-IL-15. From these results, we conclude that the antiviral activity of virus-encoded IL-15 involves both IFN-α/β and IFN-γ, and that the activity of the IFNs accounts fully for the antiviral activity of IL-15. Although previous studies have observed elevated IFN-β production associated with antiviral activity of IL-15 (Gill et al., 2005), this is the first demonstration that IFNs are critical for IL-15 mediated antiviral activity *in vivo*. Our study also shows that NK cells are the major source of IL-15-induced IFN production, but that additional cells, as yet unidentified, also contribute to IFN-β production.

In summary, IL-15 expressed by rVV infection contributed to virus clearance via direct and indirect pathways which included: i) enhanced NK cytolytic activity, ii) increased secretion of IFNs (IFN-γ, IFN-β) and downstream IFN-inducible proteins by NK cells and other cells, and iii) increased IRF-1 activity, a potent regulator of IFNs and IL-15 expressions (Fig. 8). In conclusion, the use of viral vectors encoding IL-15 in the design of vaccines could provide new therapeutic strategies aimed at boosting the innate immune response. This was recently demonstrated in mice, with the integration of human IL-15 into the

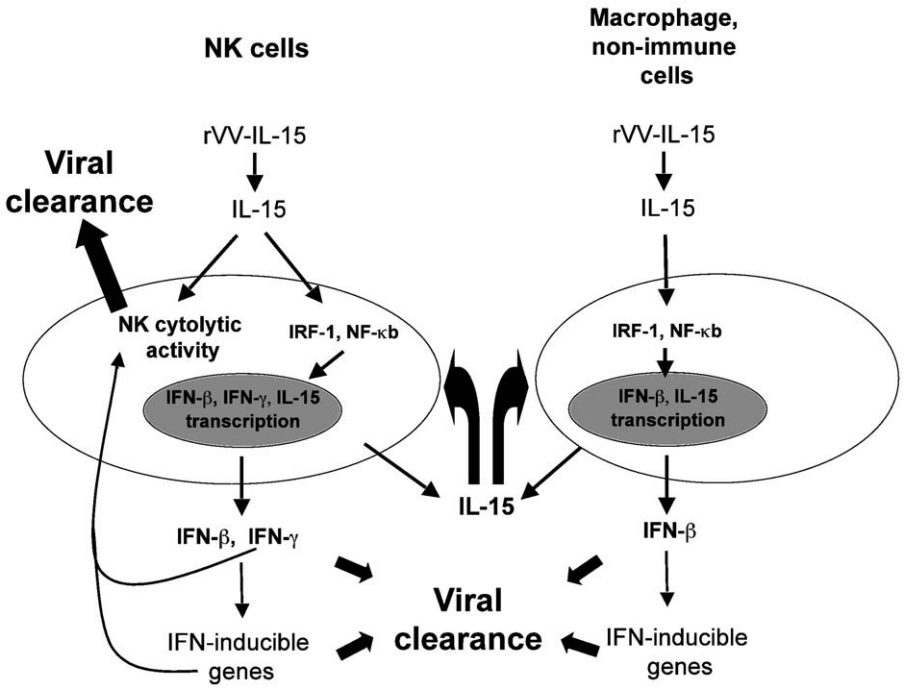

Fig. 8. VV-IL-15 mediated antiviral activity. IL-15 expressed by rVV can directly increase the cytolytic activity of NK cells. In addition, the over-expression of IL-15 triggers the activation of the transcription factors IRF-1, NF-κB and IFNs in natural killer (NK) cells, macrophages and non-immune cells. These factors mediate antiviral effects by activating NK and antigen presenting cells.

modified vaccinia virus Ankara (MVA-IL-15) that showed greater immunogenicity and efficacy than the currently licensed smallpox vaccine (Dryvax) (Perera et al., 2007).

Materials and methods

Mice

Specific-pathogen-free athymic Swiss outbred nude and C57BL/6 wild-type (WT) mice were used at 6–12 weeks of age. Mice lacking functional type I IFN receptor [IFN-α/β R GKO] and both type I and type II IFN receptors [IFN-α/β, γ R GKO] (van den Broek et al., 1995) on the 129/SvEv background were used at 6–12 weeks of age. Wild-type 29/SvEv mice were used as controls for the GKO mice. Mice deficient in the IFN-γ gene (Dalton et al., 1993) on the C57BL/6 background were used at 6–12 weeks of age. All IFN receptor gene-knockout mice were bred on the 129/SvEv mouse strain, with the exception of IFN-$\gamma^{-/-}$ mice which were bred on the C57BL/6 mouse strain. All mice were obtained from the Animal Breeding Establishment, John Curtin School of Medical Research and treated according to the Australian National University Animal Welfare guidelines.

Viruses

The neurovirulent VV strain, Western-Reserve (VV-WR), was propagated in CV-1 African green monkey kidney cells and used after purification on a sucrose density gradient, as previously described (Esposito et al., 1978). Mice were injected intravenously (i.v.) on day 0 with 10^6 plaque-forming units (pfu) of the virus. Wild-type vaccinia virus VV-WR was used to generate all rVV used in this study following previously described methods (Coupar et al., 1988; Ramshaw et al., 1987). In experiments where mice were injected with recombinant vaccinia virus (rVV) expressing murine IL-15 (VV-IL-15) or murine IL-2 (VV-IL-2), VV-X (Coupar et al., 1988) was used as the control virus. Mice were infected intravenously (i.v) or intraperitoneally (i.p) with rVVs. Prior to infection, virus stocks were diluted in phosphate-buffered saline (PBS) to the required titer and sonicated three times at 50 W for 10 s.

Cell lines

143B, YAC-1 and CTLL-2 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in DMEM (Gibco BRL) supplemented with 5% heat-inactivated fetal calf serum (FCS) (CSL, Australia), 1 mM L-glutamine (Gibco BRL), 10 mM HEPES (Gibco BRL) and antibiotics. Human osteosarcoma 143B cells were used for viral propagation of VV-X, VV-IL-15 and VV-IL-2 and for viral titrations. CTLL-2 cells were used in a proliferation assay to measure IL-15 bioactivity. YAC-1 cells were used as target cells for NK cell cytotoxicity assays.

RNA extraction and RT-PCR analysis of cytokines

Total RNA was isolated from the spleens of rVV-infected mice by standard methods using TRIzol (Gibco BRL). Reverse-transcriptase PCR (RT-PCR) was performed, as described previously (Mahalingam et al., 2001a; Mahalingam and Lidbury, 2002). Primer sequences for HPRT, IFN-β, IFN-γ and IRF-1 have been described previously (Lidbury and Mahalingam, 2000; Mahalingam et al., 1999, 2001b). Primer sequences for IL-15 and IL-2 are as follows: IL-15 sense (5′ GAACAGTGACGGATCCCCC 3′) and antisense (5′ CGGTATCGATAAGCTTGATA 3′); IL-2 sense (5′ GAGTCAAATCCAGAACATGCC 3′) and antisense (5′ TCCACTTCAAGCTCTACAG 3′). The cycle numbers used for amplification of each gene product is as follows: IFN-γ and IRF-1, 25 cycles; IFN-β and IL-15, 28 cycles; and HPRT, 23 cycles. After the appropriate number of PCR cycles, the amplified DNA was analyzed by gel electrophoresis.

ELISA for mouse IL-15

IL-15 protein was detected using the method described by Gill et al. (2005). Briefly, Maxisorp 96-well ELISA plates (Invitrogen) were coated with 100 μl of rabbit anti-mouse IL-15 (RDI) (2 g/ml) and incubated overnight at 4 °C. Plates were washed with PBS-Tween 20, blocked with 2% bovine serum albumin (BSA) at room temperature for 2 h and diluted samples (in 0.1% BSA) were incubated overnight at 4 °C. Plates were incubated with 100 μl of goat anti-mouse IL-15 (1 g/ml) (R&D Systems) for 1 h in the dark at room temperature followed by 100 μl of biotin-labeled donkey anti-goat antibody (RDI) (0.1 g/ml) at room temperature for 1 h. Plates were developed by the addition of Extravidin peroxidase conjugate (Sigma) for 45 min at room temperature and TMB microwell peroxidase (KPC) for 30 min at room temperature. Reactions were stopped by the addition of 100 μl of 1 M H_2SO_4, and plates read with an ELISA plate reader (BioRad) at 405 nm.

ELISA for mouse IFN-γ

The concentration of IFN-γ in mouse tissue samples was determined by ELISA (R&D Systems) as specified by the manufacturer.

Construction of recombinant vaccinia virus

Recombinant VV encoding murine IL-15 was made from the wild-type VV-WR virus using established procedures (Coupar et al., 1988; Mahalingam et al., 1999). The generation of recombinant viruses were based on thymidine kinase (TK) recombination, making recombinant control virus VV-X less virulent than VV-WR. Full-length murine IL-15 cDNA (Immunex Corp.) was excised from pBluescriptSK$^-$ using Sal1 and BamH1 restriction enzymes. The 545 base pair (bp) insert was gel purified and cloned into the BamH1 and Sal1 sites of the digested vector pPS7.5A (Coupar et al., 1988). Expression of mIL-15 is under the control of the early/late VV 7.5 kDa promoter P7.5, and the resulting plasmid, designated pPS7.5A-IL-15, was used in a marker rescue with VV-PR8 (HSV thymidine kinase negative virus, TK$^-$ virus) (Mahalingam et al., 1999). *In vitro* homologous recombination with VV-PR8 and pPS7.5A-IL-15 using marker rescue of the TK gene and methotrexate selection resulted in VV-IL-15 which encoded the IL-15 cDNA. Following three rounds of plaque purification, the absence of wild-type VV-WR virus and the presence of IL-15 was confirmed by PCR analysis, as previously described (Heine and Boyle, 1993).

Protein expression by recombinant IL-15 virus

Immunofluorescence assay

The expression of foreign protein by rVV was confirmed by immunofluorescence cell staining. Cell monolayers (143B cells) were grown on glass coverslips and infected with rVV at a multiplicity of infection (moi) of 0.1 for 18 h. Cells were fixed in methanol at −20 °C for 20 min, washed twice with PBS and permeabilised with 0.2% Triton-X100. Polyclonal anti-murine IL-15 antibody (R&D Systems) was added to each coverslip for a minimum of 20 min at room temperature before washing three times in PBS. FITC-conjugated anti-goat Ig (for IL-15 staining) antibody was added (1/20 dilution) for a minimum of 20 min. The fixed cells were washed and antibodies were diluted using PBSFT buffer (2% FCS, 0.2% Triton-X100 in PBS). After washing three times with PBS, the glass coverslips were quickly rinsed in water to remove salt crystals and mounted in glycerol/PBS (1:1) on glass slides. Immunofluorescence of stained cells was visualized using a BioRad Radiance 2000 Confocal Laser Scanning Microscope (CLSM) system using a Nikon Eclipse TE300 inverted microscope and a 60× oil immersion lens. Where required, transmission mode images were overlayed onto fluorescent images to enable subcellular localization of fluorescent signals to be determined.

Bioassay

IL-15 protein expression by rVV was determined using a CTLL-2 bioassay. Confluent monolayers of 143B cells were infected with VV-IL-15 and the parent virus VV-PR8 at a moi of 1 for 48 h. Supernatant was collected, centrifuged at 2000 rpm for 10 min and virus in the supernatant was removed by filtering the supernatant twice through 0.1 μm filters (Millipore). The cell filtrate was concentrated using Centricon-10 (Amicon) concentrators for 60 min in a SS34 Sorvall rotor at 5000 g before storage at −20 °C. Concentrated protein was titrated in 96-well plates before seeding with 10^4 log-phase CTLL-2 cells per well. Cells were incubated for 48 h at 37 °C with 5% CO_2 before the addition of CellTiter96® Aqueous One Solution Reagent (Promega). Cell proliferation was assessed by reading the absorbance at 490 nm 4 h after the addition of the color reagent.

Nuclear extraction

Spleen tissues were collected from groups of WT C57BL/6 mice 3 days after infection with VV-X or VV-IL-15 and cell suspensions prepared as previously described (Mahalingam et al., 2001a). Nuclear extracts were prepared according to the method described previously (Dignam et al., 1983).

Electrophoretic mobility shift assay (EMSA)

The sequences for oligonucleotides used and the EMSA protocol have been described previously (Mahalingam and Lidbury, 2002). Double-stranded oligonucleotides were labeled with ^{32}P-deoxynucleotide (Amersham) using Klenow polymerase (Roche). The reaction mixture contained nuclear extract (10 μg), 5 μg of poly(dI-dC), 1 mM dithiotreitol and 1 μl of ^{32}P-labeled double-stranded oligonucleotides (0.1 μg/μl) dissolved in the binding buffer (pH 7.9, 20 mM Tris–HCl, 30 mM NaCl, 5 μM EGTA, 50% glycerol) supplemented with 0.2 μg/ml BSA. Following incubation, electrophoresis was performed in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8) at 175 V. The gels were dried and analyzed by autoradiography.

Cytotoxicity assays

The standard ^{51}Cr-release assay for NK cell cytotoxicity was performed in triplicate for each effector to target ratio, as described elsewhere (Karupiah et al., 1990).

Determination of virus titers in organs

Ovaries and spleens were removed aseptically from groups of virus-infected mice and stored at −70 °C. Organs were thawed and homogenized in 1 ml PBS. A 100 μl aliquot of the homogenate was incubated with 100 μl trypsin (1 mg/ml) at 37 °C for 30 min, after which DMEM media with 5% FCS was added to a final volume of 1 ml. Ten-fold serial dilutions were carried out in saline with 0.5% gelatin and 100 μl of each dilution used to infect confluent 143B cell monolayers grown in six-well plates (Linbro, Flow Laboratories,) at 37 °C with 5% CO_2. After 48 h, cells were stained with 0.1% crystal violet in 20% ethanol and viral plaques counted. The limit of detection for this assay was 2 $\log_{10}$ pfu/ml.

Histology

Ovaries were collected from mice and fixed in 10% neutral buffered formalin for at least 24 h before embedding in paraffin wax. Fixed tissues were sectioned into 5 μm slices, stained with haematoxylin and eosin (H & E) and mounted onto glass slides. Analysis of stained tissue sections was performed using a Nikon CoolPix 990 Zeiss Axiophot photomicroscope.

Depletion of NK cells in vivo

A mouse monoclonal antibody, PK136, was used to deplete C57BL/6 mice of NK (NK1.1$^+$) cells. PK136 is a highly specific, anti-NK1.1$^+$ antibody (Koo et al., 1986), obtained from ascites as a gift from Dr. Guna Karupiah (Australian National University). NK cells were depleted in the mice by i.p. injection with 1 mg of PK136 diluted in PBS on days −1 and 1. Mice were infected on day 0 with 10^7 pfu virus and on day 3 the ovaries and spleens were harvested to determine viral titers. Single cell suspensions from spleens were prepared to verify NK depletion by fluorescent activated cell sorter (FACS) analysis.

Neutralization of IFN-γ and IFN-α/β in vivo

Neutralization of IFN-γ *in vivo* was performed using a monoclonal antibody (MAb) (clone XMG-6; rat IgG1). A MAb to β-galactosidase (clone GL113; rat IgG1) was used as an isotype control. Purified rabbit polyclonal antibody to murine IFN-α/β (Lee Biomolecular Research Laboratories) was used to neutralize IFN-α/β. Mice were given 1 mg each of MAb to IFN-γ or 300 U each of anti-IFN-α/β or a combination of the two antibodies i.p. on days −1 and 1. Control mice were given 1 mg of anti-β-galactosidase MAb. Mice were infected with 10^7 pfu virus on day 0 and killed on day 3.

Statistical analysis

Data is expressed as mean ± standard error of the mean (SEM). Differences between experimental groups were analyzed using either Students *t*-test (InStat 2.01 software for Macintosh) or one-way analysis of variance (ANOVA) test using GraphPad Prism4 software. Values of $p<0.05$ were considered significant.

Acknowledgments

The authors would like to thank Dr Ian Ramshaw for supporting the early work on the construction of recombinant VV expressing IL-15. We also thank Mrs Medveczky and Ms Anna Wielopolska for their technical expertise and Drs Ralph Tripp and Brett Lidbury for reading the manuscript prior to submission. SM and DAJ are recipients of the Australian NHMRC R. Douglas Wright (CDA # 303413) and NHMRC Senior Principal Research (SPRF #334013) Fellowships respectively.

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